

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :  
Akihiro KONDO et al. : Group Art Unit: 1637  
Serial No.: 09/830,652 : Examiner: Suryaprabha Chunduru  
Filed: April 30, 2001 :  
For: METHOD FOR DETECTING  
GENE AFFECTED BY  
ENDOCRINE DISRUPTOR

DECLARATION UNDER RULE 1.132

Honorable Commissioner of Patents and Trademarks  
Washington, D.C.

Sir:

I, Junichi MINENO citizen of Japan and residing in  
Kyoto Prefecture declare and say that:

1. I was graduated from Kyoto University in 1984.
2. Since 1984 up to this time, I have been an employee  
of TAKARABIO INC. (formerly Takara Shuzo Co., Ltd.), the assignee  
of the above-identified application, and have been engaged in  
research and development work on research for molecular biology  
and DNA microarray technology, and now, am the Senior Scientist  
of Center for Cell and Gene Therapy of TAKARA BIO INC.
3. I am one of the co-inventors of the  
above-identified application, and am familiar with the subject  
matter thereof.
4. I have read the Office Action mailed September

9, 2004 and the references cited therein, and am familiar with the subject matter thereof.

5. Since experimental results obtained by the present inventors show that the cited references do not teach or suggest the invention of the present application, they are set forth below.

#### EXPERIMENTAL RESULTS

##### (1) Preparation of mRNA from MCF-7 cells

###### a) Preparation of activated carbon-treated fetal calf serum

An activated carbon-treated fetal calf serum was prepared according to the method of Stanley et al. (Cell, 10:35-44 (1977)). Briefly, activated carbon (25 g of activated carbon stirred in distilled water and filtered through a filter paper; final concentration of 5% (w/v)) and dextran (final concentration of 0.5% (w/v)) were added to 500 ml of a fetal calf serum which had been inactivated by treatment at 56°C for 30 minutes. The mixture was stirred several times and allowed to stand at 4°C overnight. Then, a supernatant was collected by centrifugation at 200 rpm for 20 minutes, filtered through a paper filter, sterilized by filtration through a 0.22- $\mu$ m filter (Millipore), and stored at -20°C.

###### b) Cell culture

Cells were cultured using phenol red-free Dulbecco's modification of Eagle's medium (DMEM) (BioWhittaker) supplemented with 2 mM L-glutamine as well as 100 units/ml of

penicillin and 100 µg/ml of streptomycin (BioWhittaker) (serum-free medium) at 37°C in saturated humidity in 5% CO<sub>2</sub>/95% air.

MCF-7 cells were preincubated for 5 days. 4-octylphenol (OP) (200 nM), 4-nonylphenol (NP) (1 µM), 17-β estradiol (E2) (10 nM) or diethylstilbestrol (DES) (10 nM) was added thereto. The period of treatment with each substance was 6 hours.

Large-scale cultivation for experiments using a DNA array was carried out as follows. MCF-7 cells which had been cultured in a serum-containing medium were trypsinized, collected by centrifugation at 1000 rpm for 1 minute, and washed with the serum-free medium. The cells were suspended in the serum-free medium to single cells using a Pasteur pipette, and placed in a 10-cm culture dish at a density of  $1 \times 10^6$  cells/plate. The medium was exchanged after three days and the cultivation was further continued for two days. Each one of the above-mentioned four test substances was added to collected cells such that the concentration of dimethyl sulfoxide used for dissolving the substance was 0.1%. The cells were cultured for 6 hours.

The cells were washed with PBS, trypsinized and collected by centrifugation at 1000 rpm for 3 minutes.

#### c) Preparation of total RNA

Total RNA was prepared using StrataPrep (Stratagene) according to the manufacture's instruction manual. The concentration of the thus obtained total RNA was calculated

based on the absorbance at 260 nm (OD260) and the purity was calculated based on the ratio OD260/OD280. 16 to 31 µg of total RNA was prepared from MCF-7 cells cultured in a 10-cm culture dish with purity of 2.1.

(2) Analyses of genes using DNA array

a) Preparation of probe

3 µl of Oligo dT primer (100 pmol/µl) was added to 20 µg of total RNA prepared from cells with or without treatment with the above-mentioned substance. The mixture was subjected to heat denaturation at 70°C for 5 minutes, and then rapidly cooled on ice. 4 µl of 5 x AMV buffer, 2.0 µl of 10 x dNTP mixture (Takara Bio), 1 µl of 1 mM Cy3- or Cy5-dUTP (Amersham Pharmacia), 20 U of Ribonuclease inhibitor (Takara Bio) and 30 U of AMV (Takara Bio) were added thereto and the total volume was adjusted to 20 µl. The mixture was incubated at 42°C for 90 minutes. The Cy5-labeling was used for the control group and the Cy3-labeling was used for the test group. After treatment at 70°C for 10 minutes, the probe was purified using CentriSep.

The volume of the eluent was adjusted to 100 µl with DEPC-treated water, an equal volume of chloroform/isoamyl alcohol (24:1 (v/v)) was added thereto, the mixture was vortexed and centrifuged at 14500 rpm for 5 minutes, and the aqueous layer was recovered.

2 µl of 5 x Competitor I (Human) (Takara Bio) was added to the aqueous layer, and the mixture was subjected to ethanol precipitation. The precipitate was dissolved in 10 µl of a hybridization solution (6xSSC, 0.2% SDS, 5xDenhardt's solution,

0.1 mg/ml salmon sperm DNA). The thus obtained solution was used as a probe solution.

b) Prehybridization

A DNA array comprising at least one gene for each of the respective groups (a) to (q) according to the present invention was prepared after preparing DNA fragments for the 49 genes listed in the attached table (the letters between the parentheses correspond to the groups) according to the method as described in Example 1. A cover glass was placed on the DNA array, 10 µl of a prehybridization solution was added thereto, and incubation was carried out for 2 hours. The array was washed with 2 x SSC followed by 0.2 x SSC and centrifuged at 1000 rpm for 3 minutes for drying.

c) Hybridization and washing

The probe solution was subjected to heat denaturation at 95°C for 2 minutes, incubated at room temperature for several minutes, centrifuged at 14500 rpm for 10 minutes at 20°C to remove precipitates, and added to the DNA array covered with a cover glass. Hybridization was carried out in a hybridization chamber at 65°C overnight. After hybridization, the cover glass was carefully removed in a solution of 2 x SSC/0.2% SDS, and the array was washed twice in a solution of 2 x SSC/0.2% SDS at 55°C for 30 minutes, in a solution of 2 x SSC/0.2% SDS at 65°C for 5 minutes, and finally in 0.05 x SSC for 30 minutes for removing SDS. Centrifugation at 1000 rpm for 5 minutes was carried out for drying the DNA array. The DNA array was

subjected to measurements of Cy3 or Cy5 fluorescence from the respective spots using Affymetrix 418™ Array Scanner (Takara Bio). Gene clustering analyses were carried out based on the measured fluorescence. The results are shown in the attached figure.

The analysis data represent results of hierarchical clustering using a gene expression analysis software GeneSight™ ver. 4.0 (Biodiscovery). Specifically, the longitudinal axis on the left represents the gene cluster. The values on the left of the horizontal axis represent the Euclid distance obtained from the hierarchical clustering. Bridge with a small value indicates that, for example, it is highly possible that the genes exhibit similar expression patterns or the genes relate to each other (in a signal transduction). Compounds used for the treatments which have been reported to have endocrine-disrupting activities, 17-β estradiol (E2), diethylstilbestrol (DES), 4-octylphenol (OP) and 4-nonylphenol (NP), are shown on the right of the horizontal axis. The longitudinal axis on the right represents numbers assigned to genes selected from the groups (a) to (q) according to the present invention. The numbers correspond to those indicated in the attached table which shows the names of the genes and the groups to which the genes belong.

In the analysis data, the red color indicates that the expression of the gene is increased or promoted as a result of treatment with the compound, while the green color indicates

that the expression of the gene is decreased or suppressed as a result of treatment with the compound. The black color indicates that no data were available or no change was observed.

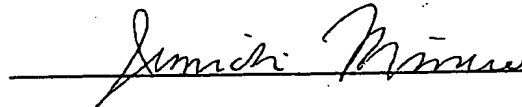
As seen from the analysis data, the genes selected from the groups (a) to (q) according to the present invention constituted characteristic gene clusters upon treatments with the compounds which have been reported to have endocrine-disrupting activities, and their gene expression patterns were characteristic. For example, the expression patterns obtained using treatments with OP and NP, which are phenols, were almost identical to each other.

Thus, according to the present invention, gene clustering analyses could actually be carried out for the four compounds which have been reported to have endocrine-disrupting activities (i.e., E2, DES, OP and NP) and, for example, a signal transduction pathway characteristic of phenolic compounds could be analyzed by carrying out gene clustering analyses using the phenolic compounds OP and NP.

6. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application

or any patent issuing thereon.

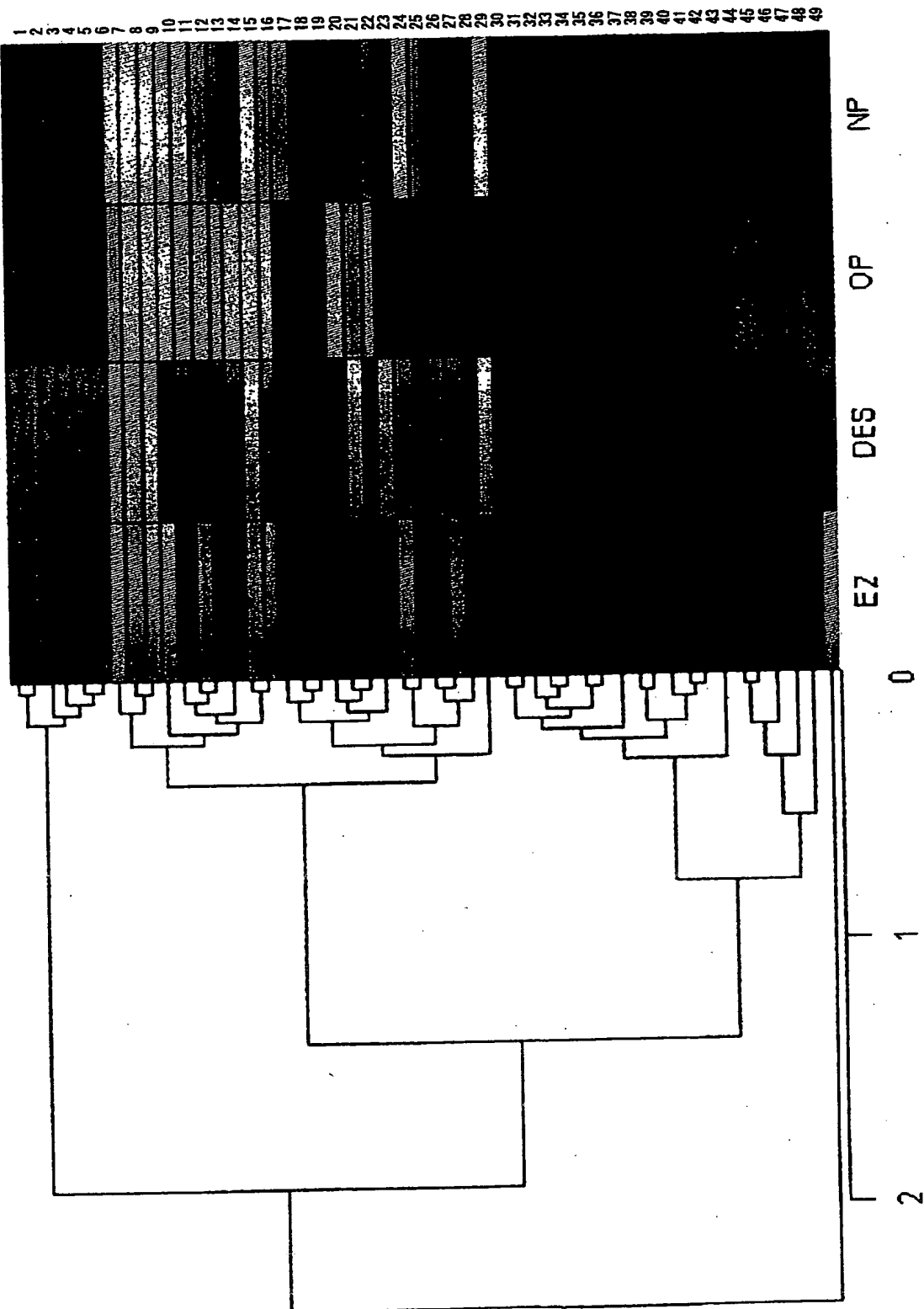
This 2 day of November, 2004

A handwritten signature in cursive script, appearing to read "Junichi Mineno", is written over a horizontal line.

Junichi MINENO



Gene Clusters



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2.599

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- No.
- 1 (a) p300/CBP
  - 2 (a) ACTR
  - 3 (a) RIP1 40
  - 4 (a) TRIP1
  - 5 (d) PDGF receptor
  - 6 (d) VEGF receptor
  - 7 (a) N-CoR/SMRT
  - 8 (f) retinoblastoma-binding protein(RBP)
  - 9 (m) VEGF-B precursor
  - 10 (f) CLK-2
  - 11 (g) k-ras oncogene
  - 12 (h) 8 regulator bcl-w
  - 13 (k) dishevelled 2(DVL)
  - 14 (h) BAD protein(BCL-2 binding component 6)
  - 15 (l) paxillin
  - 16 (q) TGF-beta3
  - 17 (c) SOX9
  - 18 (j) DNA Topoisomerase I
  - 19 (o) APC
  - 20 (h) induced myeloid leukemia cell differentiation protein MCL-1
  - 21 (p) ras like small GTPase TTF
  - 22 (p) rho-associated coiled-coil containing protein kinase p160ROCK
  - 23 (j) IGFBR2
  - 24 (f) growth factor receptor-bound protein2(GRB2) isoform
  - 25 (g) STAT5B
  - 26 (l) integrin alpha-3 chain
  - 27 (q) HBEGF(DTR)
  - 28 (l) integrin beta4
  - 29 (l) CCK4(PTK7)
  - 30 (a) TIF2
  - 31 (g) c-Myc
  - 32 (f) cdc25C(M-phase inducer phosphatase3)
  - 33 (h) cdc42 homolog(G25K)
  - 34 (p) cdc42 GTPase-activating protein
  - 35 (l) integrin-like kinase (ILK)
  - 36 (n) urokinase-type plasminogen activator receptor
  - 37 (q) cytokine humig (interferon-gamma-induced monokine)
  - 38 (a) ARA70
  - 39 (b) JNK2
  - 40 (g) c-fos
  - 41 (h) cytotoxic ligand TRAIL receptor
  - 42 (j) DNA-PK catalytic subunit
  - 43 (g) Bax a,b,g
  - 44 (e) cytokeratin 13 (CK13)
  - 45 (e) profilin
  - 46 (e) cytokeratin 18 (CK18)
  - 47 (l) beta 3-endonexin
  - 48 (h) PIG12
  - 49 (b) BMK a,b,g

